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TNF-receptors on human peritoneal mesothelial cells: Regulation of receptor levels and shedding by IL-1α and TNFα

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TNF-receptors on human peritoneal mesothelial cells: Regulation of receptor levels and shedding by IL-1α and TNFα. Human peritoneal mesothelial cells (HPMC) respond to tumor necrosis factor α (TNFα) by releasing various cytokines that may activate the endothelium and induce recruitment of leukocytes during peritonitis. We characterized the receptors for TNF on HPMC to elucidate their functions in peritonitis. Scatchard analysis determined the presence of 70×10^3 TNF receptors/cell with a kDa of 0.44 nM. TNF receptor 1 (TNF-R1, p55) and TNF-R2 (p75) mRNA were demonstrated by reverse-transcriptase-PCR (RT-PCR). TNF-R1 protein was solely detected by flow cytometry (FCM). Interleukin-1α (IL-1α) induced down-regulation of TNF-R1. This was concomitant with accumulation of soluble TNF-R1 (sTNF-R1) detected by specific ELISA. LPS had a lower TNF-R1-shedding activity while TNFα did not induce shedding. The IL-1-induced-sTNF-R1-shedding was suppressed by the protein-kinase-A (PKA) inhibitor, H-8, or by H-7, the inhibitor of both PKC and PKA, but not by the specific PKC inhibitor GF. These experiments suggest a role for PKA in the IL-1-shedding signal. No change in TNF-R1 mRNA levels was observed after IL-1α or TNFα stimulation while TNF-R2 (p75) mRNA basal levels transiently increased three to fivefold, reaching a peak after four hours followed by an accumulation of sTNF-R2 in the supernatant. Our data suggest that the main receptor expressed on HPMC is TNF-R1. Down-regulation and shedding of TNF-R1 induced by IL-1, and the transient expression of TNF-R2 induced by IL-1 and TNF, may regulate the responses to TNF by HPMC. These results may be important in understanding the inflammatory process of peritonitis were TNF plays a major role.

Mesothelial cells constitute the main cell population of the peritoneal membrane. Much evidence has been gathered indicating that in continuous ambulatory peritoneal dialysis (CAPD) these cells function not only as a barrier limiting the traffic of solutes, but also play a central role in the inflammatory/immune response of the peritoneum [1–4].

In response to contact with bacteria and bacterial products, macrophages produce tumor necrosis factor α (TNFα) and interleukin-1 (IL-1) [4, 5]. These cytokines initiate an inflammatory cascade causing vasodilation and chemotaxis, leading eventually to recruitment of leukocytes. In the initial phase of peritonitis, only a small number of resident macrophages are present in the peritoneum. Thus, in order to stimulate recruitment of leukocytes, the macrophage-derived pro-inflammatory mediators must be amplified. Because the mesothelial cell layer is in close proximity to the underlying peritoneal capillaries, it is the cell population most likely to be involved in the transduction of these signals to the endothelium. This assumption is supported by the finding that TNFα alone, or in synergism with IL-1 and endotoxin, induce production of IL-1α and IL-1β by HPMC [1]. These cytokines are potent endothelial cell activators known to induce expression of adhesion molecules, chemoattractants and other inflammatory products. This process eventually leads to the activation and recruitment of leukocytes in the inflamed tissue. Furthermore, it has been shown that in response to TNF, HPMC produce IL-6 [2], the neutrophil chemoattractant-IL-8 [3, 4] as well as various other pro-inflammatory agents, all of which may synergize in the process of activation and transmigration of leukocytes to the site of injury. Thus, the response to TNF by mesothelial cells is a critical event during peritonitis.

TNF is a pleothropic factor that is influential in mediating many pathological conditions such as tissue necrosis and fibrosis [6]. TNF increases oxygen radical production by polymorphonuclear cells, which in turn cause tissue destruction [7]. Activated neutrophils were shown to be destructive to mesothelial cells [8]. This is probably the first stage in the peritoneal scarring and fibrotic process promoted by TNF-induced proliferation of fibroblasts.

TNFα acts by binding to receptors on target cells. Two TNF receptors were cloned: type 1 receptor with a 55 kDa transmembrane protein (TNF-R1, p55) and type 2 with 75 kDa transmembrane protein (TNF-R2, p75) [9, 10]. The p55 receptor is ubiquitous, whereas the p75 receptor is restricted mainly to cells of hematopoietic origin [11]. Although many cells express only one of the two TNF receptors, some highly responsive cells such as vascular endothelial and natural killer (NK) cells often bear both receptor subtypes [12, 13].

Studies on regulation of TNF bioactivity have shown the existence of biological mechanisms restricting the potentially adverse effects of this cytokine. It is believed that one such mechanism is instrumental in the formation of soluble forms of TNF receptors (sTNF-Rs) that specifically bind TNF so that adhesion to the cell surface TNF receptor is avoided [11, 14, 15]. The released receptors intercept TNF and thereby prevent target cell activation.

Restriction of TNF activity in the peritoneum by shedding of TNF receptors may have an important role in preventing membrane damage and fibrosis. Conversely, shedded soluble TNF-receptors (sTNF-Rs) seem to stabilize the bioactive trimeric structure of TNF [16]. There are two types of sTNF-Rs derived from the shedding of the extracellular domain of the cytoplasmic

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TNF receptors [17]. Both were found at elevated levels in patients with sepsis, cancer, febrile illness, and chronic renal failure [18–21]. Elevated levels of TNFα as well as the presence of both soluble receptors were found in the peritoneal effluent of patients undergoing CAPD during peritonitis. Peak levels of TNFα were observed at day one and sTNF-Rs at day two [22].

To assess the peritoneal inflammatory response to TNF, we examined human peritoneal mesothelial cells (HPMC) in culture for TNF receptor membrane expression, RNA levels, and regulation of shedding.

Methods

Cell preparation and characterization

Human peritoneal mesothelial cells (HPMC) were isolated from portions of omentum of approximately 6 cm^2 obtained during elective abdominal surgery [23]. The pieces were washed in phosphate buffered saline (PBS) and then sliced into portions of approximately 1 mm^3. The disaggregation of the tissue was performed by continuous stirring of the tissue for one hour at 37°C in a 50 ml centrifuge tube in the presence 0.2% collagenase A (Boehringer Mannheim, Mannheim, Germany) in M199 medium containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and Biogro 2™. This supplemented medium is used in all the experimental protocols described below and is referred as M199 medium. The cells were washed three times in PBS and then seeded in a 75 cm^2 tissue culture flask (Bibby, Corning, NY, USA) with M199 medium. The cells were cultured in an atmosphere of 5% CO_2 at 37°C. After reaching confluence, cells were trypsinized (trypsin-EDTA, solution B), washed and diluted in fresh medium. Most experiments were performed during the third passage. The cells in this passage had a uniform appearance and micovilli were noted to be present. Immunocytochemical analysis using the avidin biotin complex technique with three intermediate filament antibodies demonstrated that all the cells coexpressed vimentin (-CKVNS; DPC, Los Angeles, CA, USA) and cytokeratins (DPC-CKKHS, stratifying keratin, DPC-CKKLS, simple keratin), which is characteristic of mesothelial cells [23]. The cultured cells did not contain cells expressing CD 68 (patts-M-814; Dako, Copenhagen, Denmark), a macrophage/monocyte marker, or HLA-DR (Becton-Dickinson, Mountain View, CA, USA), and were Factor VIII negative. All tissue culture reagents contained less than 0.025 ng/ml of endotoxin as tested by *Lumulus Amebocyte* lysate (LAL) assay (Atlas Bioscan, Bognor Regis, UK). Unless otherwise stated, all tissue culture reagents were obtained from Biological Industries (Bet Haemek, Israel).

Recombinants

Recombinant human IL-1α (rhIL-1α) was a generous gift of Hoffman-La Roche (Nutley, NJ, USA). Recombinant human TNFα (rhTNFα) was obtained from Genentech, Inc. (San Francisco, CA, USA).

Radio receptor assay

Scatchard analysis. HPMC (2.5×10^4 cells/well) were seeded in 24 well plates (Bibby) and then incubated for 48 hours until confluence. The M199 medium was then replaced and the cells were incubated in duplicate, with or without rhIL-1α (100 U/ml) for 45 minutes Then the medium was replaced with 300 μl of cold medium containing 1 nM of ^{125}I-TNFα (500 Ci/mmol) (Amersham, Buckinghamshire, UK) and in the presence of varying dilutions of unlabeled rhTNFα (0.01 to 500 ng/ml). The cells were incubated on ice for two hours, washed three times with cold PBS, harvested by trypsin-EDTA and the incorporated radioactivity was counted in a γ-counter. In each plate, cells in two unlabeled wells were counted using a hemocytometer for the final definition of cell number per well. The affinity and number of receptors per cell were calculated using Scatchard analysis.

Down-regulation of TNF receptors. The decline of membrane TNF receptor, after IL-1 stimulation, was defined by single point radio receptor assay, after incubation with rhIL-1α (100 U/ml) for various lengths of time, washing with cold PBS and incubation with saturating amount of ^{125}I-TNFα (2 nM) for two hours on ice. Background radioactivity was defined in the presence 100-fold "cold" rhTNFα.

Activation protocols

HPMC (5×10^4 cells/well) were seeded in 12 well plates (Bibby) and then incubated for 48 hours until confluence. Cells were washed twice with medium and incubated for various lengths of time with 1 ml of M199 medium containing various concentrations of rhTNFα, rhIL-1α, *E. coli* 055:B5 lipopolysaccharide (LPS; Sigma) PMA or combination of rhTNFα and rhIL-1α as indicated in the **Results** section. After incubation, the supernatants were collected, frozen at −20°C, and subsequently assayed for sTNF-R1 and sTNF-R2. During the time course of the experiments, the various stimuli were not toxic to HPMC, as assessed by lactate dehydrogenase (LDH) release from the cells, and did not notably alter the cell number between the different treatments, as assessed by determination of total LDH activity in lysates. In each experimental plate, cells of two wells were counted for the final definition of cell number per well.

For the mRNA study, similar incubation protocols were used. At the end of incubation, supernatants were removed, cells were washed once with cold PBS and lysed with 1 ml of Tri Reagent™ (Molecular Research Center, Inc., Cincinnati, OH, USA), transferred to Eppendorf tubes and frozen at −70°C.

Shedding inhibition by protein kinase (PK) inhibitors

In the experiments with protein kinase inhibitors, cells were incubated one hour with 25 μM H-7 (K_i for PKA = 3 μM and for PKC = 6 μM), 25 μM H-8 (K_i for PKC = 1.2 μM) or 6 μM Bisindolylmaleimide I (GF, K_i for PKC = 0.01 μM). All inhibitors were purchased from (Calbiochem, San Diego, CA, USA) then rhIL-1α (100 U/ml) or PMA (10^{-7} M) were added for an additional six hours. Supernatants were collected and assayed for sTNF-R1. Preliminary experiments were performed to find the optimal non-toxic concentration of each inhibitor.

Flow cytometry (FCM) quantification of TNF receptor

Along with the information obtained from the radio receptor assays we evaluated the type and relative numbers of TNF-Rs by FCM (FACStar; Becton Dickinson, San Jose, CA, USA). Prior to FCM evaluation, HPMC (~10^6 cells/in 25 cm^2 flask) were incubated with or without rhIL-1α (100 U/ml) for various lengths of time, then harvested using cold EDTA solution on ice. HPMC were labeled by mouse anti-human TNF-R1 (10 μg/ml) antibodies or rat anti-human TNF-R2 antibodies (10 μg/ml; Genzyme, Cambridge, MA, USA) 30 minutes on ice in PBS containing 2%

Table 1. Amplification primers

Gene		Oligonuclotide sequence	Product size	References
TNF-R1	5′ primer	5′-GTGCTGTTGCCCCTGGTCAT-3′	543 bp	[9]
	3′ primers	5′-TTCTGCAGCTCCAGCCG-3′		
TNF-R2	5′ primers	5′-CAGGTCGCATTTACACCCTAC-3′	522 bp	[10]
	3′ primer	5′-CTGCATCCATGCTTGCATTCC-3′		
β-actin	5′ primer	5′-GGGTCAGAAGGATTCCTATG-3′	237 bp	[24]
	3′ primer	5′-GGTCTCAAACATGATCTGGG-3′		

bovine serum albumin (BSA; Biological Industries) and 0.01% sodium azide. The cells were washed twice in PBS/BSA and stained with fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG or rhodamine-labeled anti-rat IgG (Jackson Immuno Research Laboratories, West Grove, PA, USA) accordingly. The cells were subsequently washed twice in PBS/BSA and fixed in 2% paraformaldehyde (Merck, Darmstadt, Germany) and stored at 4°C until analysis. Flow cytometric measurements were based on 10,000 cells and displayed as frequency distribution histograms. For each experiment, clustering of cells without specific antibody labeling was evaluated and limited by borders that included 90% of the cells. After labeling, cells outside these borders were counted as positive for TNF receptors, and analyzed for mean fluorescence.

Soluble TNF receptors assays

We tested the presence of sTNF-R1 and R2 in cell supernatants using specific enzyme-linked immunoassays (ELISAs, R&D Systems, Minneapolis, MN, USA). Each sample was tested in duplicate using the standard protocol of the assay. Both ELISAs for sTNF-R1 and R2 have no significant cross-reactivity or interference with known cytokines, except for recombinant human TNFα (rhTNFα), which interfered with the sTNF-R1 assay in quantities above 5 ng/ml. To avoid this interference, supernatants containing rhTNFα at levels of 5 ng/ml or higher were diluted before the assay. The ELISAs sensitivity to sTNF-R1 and R2 in culture media samples were 1.0 and 0.5 pg/ml, respectively.

Analysis of RNA by reverse transcription-PCR

RNA extraction. Total RNA was extracted from the cells according to the Tri Reagent™ protocol. Cells were lysed with 1 ml of Tri Reagent™ upon activation and frozen at −70°C as described above. The frozen tubes were thawed and 200 μl of chloroform was added to each tube. The tubes were then vortexed for 15 seconds and after five minutes at room temperature were centrifuged for 30 minutes at 4°C in a Microfuge E (Beckman Instruments Inc., Palo Alto, CA, USA). From each tube 450 μl of the upper phase were transferred to a new tube and then the RNA was precipitated by adding 450 μl of isopropanol. Following incubation for 45 minutes on ice, 15 minutes of centrifugation at 4°C, the pellet was briefly washed by 75% ethanol then dried and resuspended in 0.3 M sodium acetate and 2.5 volumes of cold ethanol. The RNA was kept at −70°C.

cDNA preparation. The RNA was precipitated and dried. Each sample was then dissolved in 12 μl of water and heated for 10 minutes to 65°C. To each RNA sample we added a reverse transcriptase reaction mixture containing 1 μl of Moloney murine leukemia virus-reverse transcriptase (MMLV-RT, 200 U/μl; Gibco BRL, Gaithersburg, MD, USA), 4 μl of 5x reverse transcriptase buffer (Gibco BRL), 0.5 μl DTT (0.1 M, Gibco BRL), 0.5 μl RNase inhibitor (40 U/μl, Boehringer Mannheim), 1 μl oligo-d(T) 12 to 18 mer (40 pmol/μl, Boehringer Mannheim), and 1 μl dNTP's (2.5 nmol/μl each nucleotide, Boehringer Mannheim). The tube was incubated for one hour at 37°C, then the volume of each sample was adjusted to 60 μl and the enzyme was inactivated by incubation for 10 minutes at 65°C.

PCR. Samples of the cDNA preparation were analyzed for specific cDNA of TNF-R1, TNF-R2 and β-actin by PCR amplification using specific primers (Table 1). Five microliters of reverse transcription product was added to 45 μl of PCR reaction mixture containing 32.75 μl H_2O, 2.5 μl 5′ primer (20 μM), 2.5 μl 3′ primer (20 μM), 2 μl dNTPs (2.5 nmol/μl each nucleotide; Boehringer Mannheim), 5 μl of 5× reaction buffer (Perkin Elmer Cetus, ILS Ltd., London, UK) and 0.25 μl *Taq* polymerase (2.5 U, Amplitaq©; Perkin Elmer Cetus). A negative control consisting of the reaction mixture without cDNA was included with each run. The cDNA was amplified for 25 (β-actin), 30 (TNF-R1) or 35 (TNF-R2) cycles in a temperature cycler: 90 seconds at 94°C, then 10, 15 or 20 cycles of 45 seconds at 94°C, 1½ minutes at 60°C and one minute at 72°C. The last 15 cycles were the same, except that each cycle of the 72°C step was prolonged by five seconds. Eight microliter samples from the various amplified cDNAs, together with 8 μl of the corresponding β-actin amplified cDNA were loaded on the same lane and then run on agarose gels (2%) containing 0.5 μg/ml ethidium bromide. As a DNA size marker we used the "123 bp ladder" (Gibco BRL) or "100 bp ladder" (Promega, Madison, WI, USA). Quantitative evaluation of the PCR product was performed by video densitometry of the agarose gel using the UVP GDS 5000 system and software (UVP Inc., San Gabriel, CA, USA). For each sample the emission ratio of TNF-R and β-actin was calculated. To facilitate comparison between different assays, the maximal emission ratio value of each experiment was normalized to 1, and all values of other samples were normalized accordingly.

Statistics

Analysis of variance (ANOVA) was used to compare TNF receptor production between two groups. *P* values below 0.05 were considered significant.

Results

Receptor type and affinity

TNF receptors on HPMC were initially studied by radio receptor assays. HPMC were incubated with ^{125}I-TNFα and various concentrations of unlabeled ("cold") rhTNFα. As shown in Figure 1, the Scatchard analysis demonstrated the presence of $70 \pm 16 \times 10^3$ receptors/cell with an affinity of $K_d = 0.44 \pm 0.12$ nM ($N = 5$). The TNF-Rs affinity that we found in HPMC match the reported affinity of the type 1 TNF receptor [25]. Indeed, by

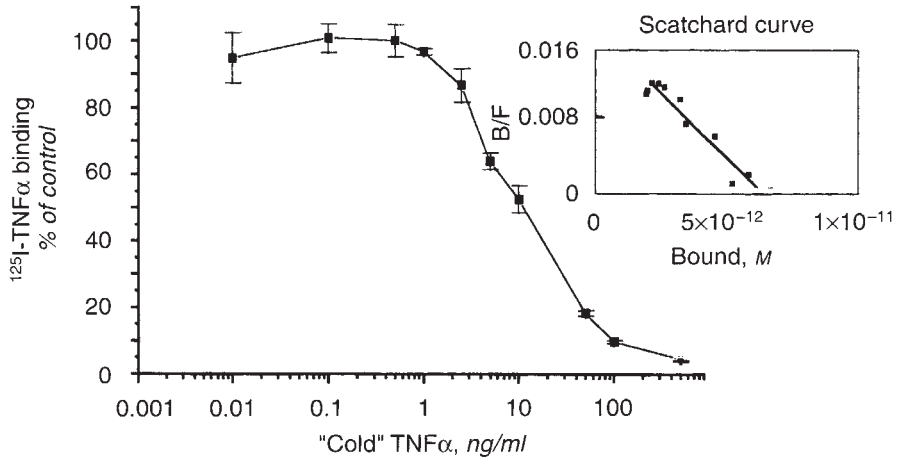

Fig. 1. *Scatchard analysis of TNF receptors on HPMC.* HPMC were incubated on ice for two hours with medium containing 1 nM of ^{125}I-TNFα (500 Ci/mmol) and in the presence of varying dilutions of unlabeled rhTNFα (0.01 to 500 ng/ml). The incorporated radioactivity was counted in a γ-counter. The affinity and number of receptors per cell were calculated using Scatchard analysis resulting in 70 ± 16 × 10^3 receptors/cells with a K_d of 0.44 ± 0.12 nM (mean ± SD of 5 independent experiments on cells from different donors). The baseline (100%) was ^{125}I-TNF bindings to unstimulated cells. In all experiments the total counts were ~10^5 cpm, with binding of ~2,000 cpm, and background of less than 5% of the binding.

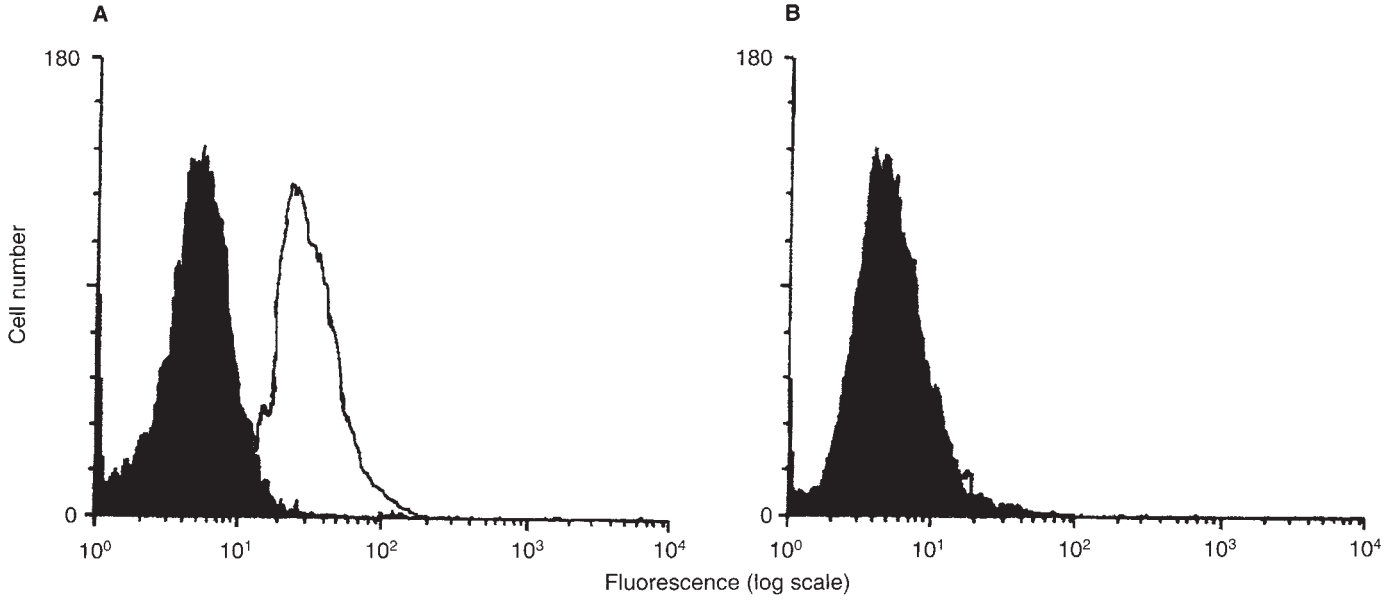

Fig. 2. *Flow cytometry (FCM) of TNF-Rs on resting HPMC.* HPMC were labeled by mouse anti-human TNF-R1 antibodies or rat anti-human TNF-R2 antibodies and stained with FITC-labeled anti-mouse IgG or rhodamine-labeled anti-rat IgG accordingly. The dark areas indicate the background fluorescence obtained from secondary antibodies alone; the lines indicate specific staining with FITC labeled antibodies for TNF-R1 (**A**) or rhodamine-labeled for TNF-R2 (**B**). The data are representative experiment out of 3 performed on cells from different donors.

using FCM analysis we revealed that over 90% of HPMC were positively stained with specific antibodies toTNF-R1, while TNF-R2 could not be detected (Fig. 2).

Number of receptors following IL-1α stimulation

Stimulation of HPMC with rhIL-1α resulted in a relatively minor increase in the number of receptors (first 10 to 15 min), followed by a decline reaching a minimum after 45 minutes and a slow recovery. This was demonstrated by single point radio receptor assay (Fig. 3) and by FCM (Fig. 4), using specific antibodies to TNF-R1. Scatchard analysis performed 45 minutes following IL-1 stimulation (not shown) confirmed that reduced binding of ^{125}I-TNFα was the outcome of a decrease in the number of receptors (from 70 × 10^3 to 18 × 10^3 receptors/cell) and not the result of reduced affinity (K_d = 0.44 ± 0.12 on quiescent cells and 0.36 ± 0.09 following IL-1). In contrast to TNF-R1, FCM analysis failed to demonstrate the presence of TNF-R2 following IL-1 stimulation up to 90 minutes (Fig. 4) or after longer exposure to rhIL-1α (100 U/ml) or rhTNFα (10 ng/ml) up to eight hours (not shown).

Shedding of TNF-R1 from HPMC

The IL-1-induced decline of TNF-R1 cell-membrane was associated with shedding of the receptors from HPMC. HPMC were exposed for various lengths of time either to rhIL-1α (100 U/ml) or to phorbol 12-myristate 13-acetate (PMA, 10^{-7} M). Cell supernatants were collected and assayed for the presence of soluble TNF-receptors (sTNF-Rs) shedded to supernatants from HPMC using ELISA for sTNF-R1 and sTNF-R2. As depicted in Figure 5, following IL-1 stimulation, an increase in TNF-R1

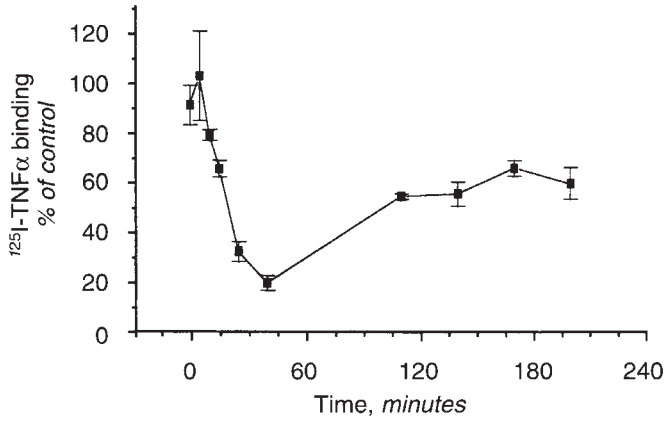

Fig. 3. *Binding kinetics of ^{125}I-TNFα to IL-1 stimulated HPMC.* HPMC were incubated rhIL-1α (100 U/ml) for various lengths of time and then incubated with saturating amounts of ^{125}I-TNFα (2 nM) for two hours on ice. Background radioactivity was defined in the presence 100-fold "cold" rhTNFα. The detailed labeling and harvesting protocol is described in Figure 1. The data represent the mean ± SD of 3 independent experiments performed on cells from different donors.

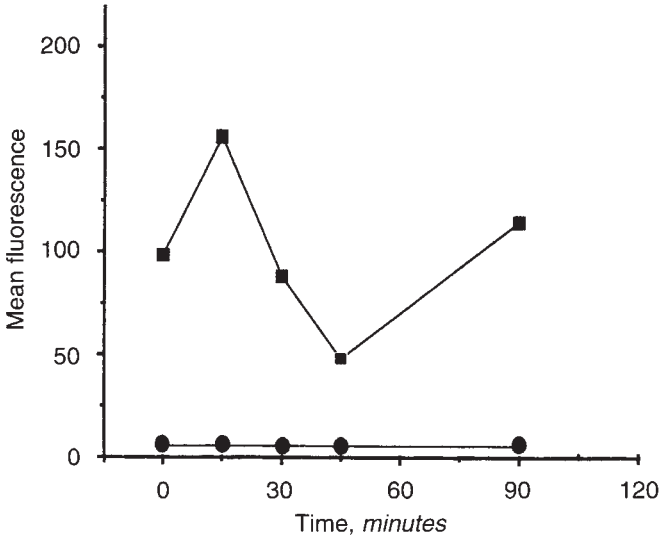

Fig. 4. *TNF receptors following IL-1 stimulation; kinetics study by FCM.* HPMC were incubated with rhIL-1α (100 U/ml) for various lengths of time, then as described in Figure 2, HPMC were labeled with mouse anti-human TNF-R1 (■) and rat anti-human TNF-R2 (●) antibodies. The data are a representative experiment out of three performed on cells from different donors, displaying the mean fluorescence of the stained cells less cells' background.

shedding could be detected after 10 minutes, reaching a plateau at 60 minutes (during this 1 hr period we calculated that 32.3×10^3 receptors/cell were shed). The ability of PMA to act as a strong shedding inducer of TNF-Rs has been reported previously in other cell systems [26]. This concurs with our findings that the accumulation of sTNF-R1 in the supernatants of HPMC could be detected very shortly after PMA stimulation. The duration of the shedding process, following PMA stimulation was prolonged, reaching a plateau at six hours (not shown). Interestingly, sTNF-R1 is continuously shedded from mesothelial cells, at a low

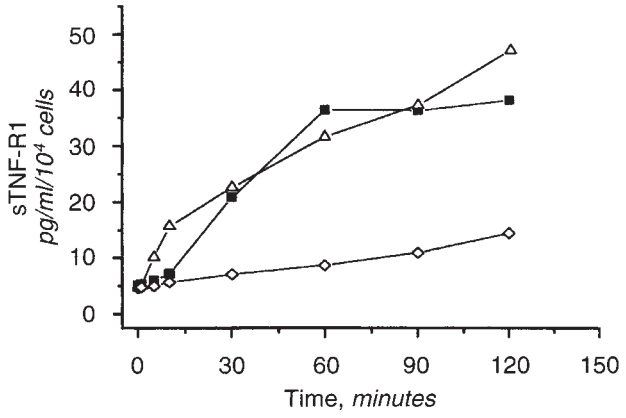

Fig. 5. *Shedding of sTNF-R1 by HPMC, time course.* HPMC (5×10^4 cells/well) were seeded in 12 well plates and then incubated for 48 hours until confluence. Cells were washed twice with medium and incubated for various lengths of time with medium alone (◇) or medium containing rhIL-1α (■, 100 U/ml), or PMA (△, 10^{-7} M). After incubation, supernatants were assayed for sTNF-R1 by ELISA. In each experimental plate, cells of two wells were counted for the final definition of cell number per well. The data are the mean of representative experiment out of 5 performed on cells from different donors. Standards deviation did not exceed 10% of the mean.

basal rate even without stimulation (7.8×10^3 receptors/cell/hr. Following stimulation induced by IL-1 or PMA the rate of shedding increased for a limited time (~1 hr for IL-1 stimulated cells or 5 hr for PMA) and then returned to the rate observed in unstimulated cells. The spontaneous shedding found in quiescent cells was not followed by changes in the number of receptors. This may indicate that there is a constant turnover of cell surface receptors so that shed receptors are continuously being replaced by newly synthesized ones. Spontaneous shedding was not related to endotoxin contamination (> 0.025 ng/ml) or background production of IL-1 (IL-1α and -β could not be detected in quiescent cells).

As seen in Figure 6, the range of response of HPMC to rhIL-1α was between 1 to 100 U/ml. In contrast, rhTNFα, within the range of 1 pg/ml to 10 ng/ml, did not induce TNF-R1 shedding. LPS at a concentration of 1 μg/ml showed only minor shedding activity, while higher or lower concentrations did not induce any shedding of significance.

In spite of the fact that we could not demonstrate the presence of TNF-R2 on HPMC-cell membranes, using the FCM technique, some low concentrations of sTNF-R2 accumulated in the supernatants of IL-1 or PMA stimulated cells (Fig. 7). The maximal sTNF-R2 levels after IL-1 or TNF stimulation reached only ~1/20 of sTNF-R1 levels under the same conditions (~5 pg/ml/10^4 cells following 22 hr of IL-1 stimulation). Detectable levels of sTNF-R2 were found only 6 to 7 hours following rhIL-1α (100 U/ml) or rhTNFα (5 ng/ml) stimulation or three hours after PMA addition (10^{-7} M). In contrast to sTNF-R1, no detectable levels of sTNF-R2 could be found in the supernatants of unstimulated cells.

The kinetics of messenger RNA levels were performed by RT-PCR. HPMC were stimulated for various lengths of time with rhIL-1α (100 U/ml) or rhTNFα (10 ng/ml). RNA was extracted, reverse transcribed and then amplified by PCR using specific primers for TNF-R1, TNF-R2 and for the housekeeping gene

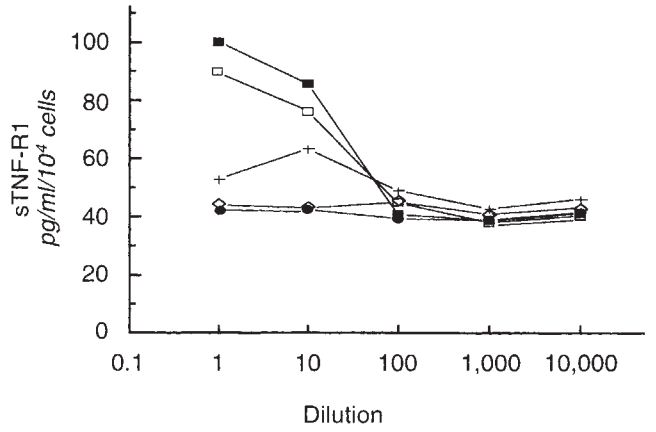

Fig. 6. *Shedding of sTNF-R1 by HPMC; dose response.* HPMC in 12 well plates were incubated for 16 hours with medium alone (◇) or medium containing 1:10 dilutions of rhIL-1α (■, 100U/ml), rhTNFα (●, 10 ng/ml), LPS (+, 10 μg/ml) or a combination of rhTNFα and rhIL-1α (□). After incubation, supernatants were assayed for sTNF-R1 by ELISA. The data are the mean of representative experiment out of 3 performed on cells from different donors. The SDS did not exceed 10% of the means.

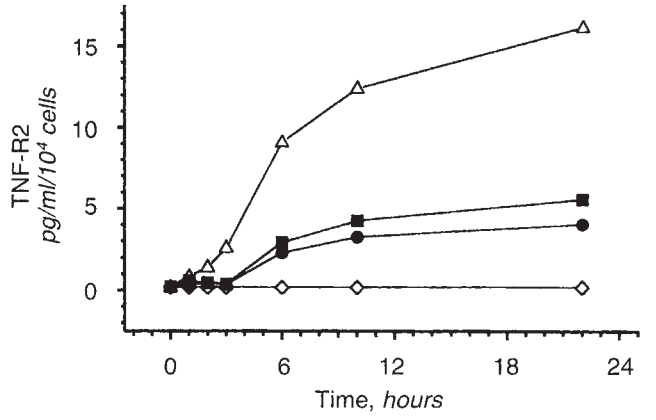

Fig. 7. *Shedding of sTNF-R2 by HPMC; time course.* HPMC in 12 wells were incubated for various lengths of time with medium alone (◇), or medium containing rhIL-1α (■, 100 U/ml), rhTNFα (●, 5 ng/ml), or PMA (△, 10^{-7} M). After incubation, supernatants were assayed for sTNF-R2 by ELISA. The data are the mean of one representative experiment out of 3 performed on cells from different donors. The SDS did not exceed 10% of the means.

β-actin as a reference. PCR amplification products were size-fractionated on agarose gels and quantitated by video densitometry. The densitometry results are the mean TNF-R/β-actin ratio of the indicated number of experiments. The number of PCR cycles for each set of primers was carefully adjusted in each experiment to the exponential phase of amplification, thus permitting comparison of of mRNA levels in the samples. In preliminary studies we also found a linear dose-response of the various PCR products to increasing doses of cDNA.

Typical time courses for TNF-R1 following IL-1 stimulation are shown in Figure 8. TNF-R1 mRNA could be demonstrated by a single band of 543 bp in unstimulated cells. No changes were observed in TNF-R1 mRNA levels following IL-1 stimulation for the indicated times. The same results were observed following TNFα stimulation or shorter IL-1 or TNF exposure (5 to 60 min, not shown). As shown in Figure 9, in contrast to the abundant TNF-R1 mRNA levels, relatively low TNF-R2 mRNA levels could be detected by a single band of 522 bp in unstimulated HPMC. At least five additional PCR cycles were needed to detect TNF-R2 from the same cDNA samples, as opposed to that needed to detect the TNF-R1 message. IL-1α and TNFα induced a short transient increase (four- to five-fold) in TNF-R2 levels. Peak levels were observed after four hours, and then there was a sharp decline that reached the basal level 24 hours after stimulation.

As shown in Figures 5 and 7, the synthetic protein kinase C (PKC) activator-PMA was found to be a strong inducer of TNF-Rs shedding from HPMC. To further elucidate the role of PKC as well as of other PKs in the signal transduction induced by IL-1α, experiments were performed using specific PK inhibitors. As shown in Figure 10, H-7 the inhibitor for PKC/PKA and H-8 the PKA inhibitor, significantly inhibited, in a similar pattern, both IL-1 and PMA induced shedding. By contrast, GF, a specific PKC inhibitor effectively blocked PMA shedding activity but had no significant effect on shedding induced by IL-1.

Discussion

A major clinical problem associated with continuous ambulatory peritoneal dialysis is the relatively high incidence of bacterial peritonitis often related to peritoneal fibrosis and loss of filtration [27, 28]. The mesothelium may have a central role in transducing the primary TNFα inflammatory signals released from peritoneal macrophages to peritoneal blood capillaries that leads to endothelial activation and leukocyte infiltration. The intensity of TNF stimulatory activity may depend upon the number of receptors on the mesothelial cell surface and by TNF accessibility to receptors. Hence, we investigated membrane regulation of TNF receptors in mesothelial cells and their release as a soluble form. TNF receptors may intercept TNF and thus limit its bioavailability. The control of TNF activity is crucial. Augmented release of TNF into the peritoneum may result in an uncontrolled, excessive accumulation of neutrophils. This may result in injury to the mesothelium that subsequently leads to fibrosis [29–31].

TNF-R1 is the main receptor expressed by mesothelial cells. TNF-R1 mRNA levels were relatively high as compared to those of TNF-R2 and the presence of TNF-R1 was exclusively demonstrated by FCM using specific antibodies. The K_d of TNF receptors (0.44 nM), obtained by Scatchard plot analysis, is in accordance with previously reported TNF-R1 affinity [25]. TNF-R1 was shown to be the main receptor present in various cells [reviewed in 11]. This receptor is the mediator for most TNF activities including, cytotoxicity, antiviral activity, cell adhesion to endothelial cells and endotoxic shock [11, 32–34]. TNF and IL-1 did not induce any change in TNF-R1 mRNA levels. This might be explained by a recent study were the promoter region of TNF-R1 was compared to housekeeping gene-promoters known to regulate constant expression of non-inducible genes [35].

The fact that TNF-R2 was undetected by FCM, and that the detected affinity matched the type one receptor plus the relatively low TNF-R2RNA levels, would indicate that type 2 TNF-receptors are not present in quiescent mesothelial cells. Pursuant to IL-1 or TNF activation, HPMC kinetic experiments show a peak in TNF-R2 mRNA levels after four hours and the accumulation of sTNF-R2 proteins after 6 to 10 hours. These data imply that

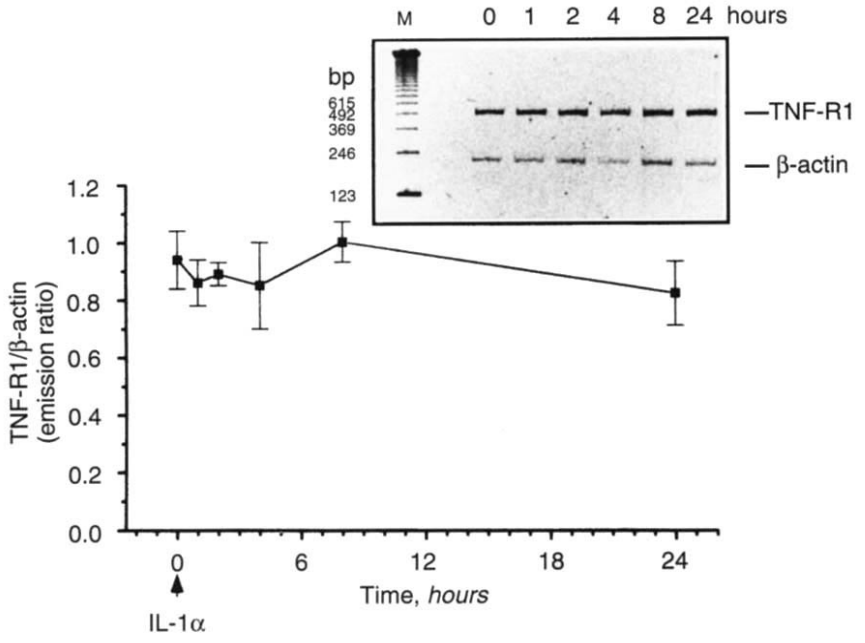

Fig. 8. *TNF-R1 RNA levels following IL-1 stimulation; time course.* HPMC were stimulated with rhIL-1α (100 U/ml) for various lengths of time. RT-PCR of RNA samples of activated HPMC were performed (25 cycles). On each lane the TNF-R1 product with the respective β-actin product as the control were loaded and then analyzed on 2% agarose gel containing ethidium bromide (0.5 μg/ml). Stimulation time and the according bands are in the insert. Three additional preparations from different donors were studied and yielded similar results. The ethidium bromide emission intensity of the bands were evaluated using a UVP GDS 5000 video system and software. The maximal emission ratio of each experiment was normalized to 1 and all the other samples were adjusted accordingly, allowing comparison between the experiments. The mean ± SD emission TNF-R1/β-actin ratio data of four experiments performed in duplicate is depicted in the plot.

TNF-R2 is *de novo*-transiently transcribed, translated and then shed. The failure to detect TNF-R2 on HPMC membrane four to eight hours after IL-1 or TNF activation, indicate that the newly synthesized receptors are probably shed very shortly after reaching the membrane. Studies on neutrophils which show the presences of TNF-R2 in cell surface have reported the immediate shedding of this receptor minutes after PMA stimulation [26]. In contrast, our study showed that low levels of soluble TNF-R2 could be detected only several hours after PMA stimulation. This further strengthens our notion that TNF-R2 is synthesized only after stimulation.

Our results indicate that TNF-R1 and TNF-R2 are independently regulated in human mesothelial cells. This is in accordance with findings reported in a study on human B lymphocytes [36]. This would imply that each of these receptors has its own specific role in transducing TNF effects. Most cytotoxic and inflammatory TNF activities are the result of TNF binding to the type 1-p55 receptor [11]. Little is known about the role of the type 2-p75 receptor. However, p75 receptor activity was found to be complementary or synergistic to TNF-R1 in experimental models [37, 38].

It is interesting to speculate whether TNF-R2, during its short period before being shed, may enhance or complement those inflammatory processes which are not performed by TNF-R1. One exciting mechanism by which TNF-R2 might access TNF activity is "ligand passing," that is, the type 2 receptor (p75) recruits TNF for signaling by the type 1 TNF receptor, as described by Tartaglia, Pennica and Goeddel [39].

Following IL-1 stimulation, a fast decline in ^{125}I-TNFα cell binding was observed with lowest binding activity at 45 minutes. On the basis of Scatchard analysis performed after 45 minutes of IL-1 stimulation and FCM experiments with anti-TNF-R1, we concluded that the decline of ^{125}I-TNFα binding was due to down regulation of type 1 TNF receptors. The kinetics of receptors down regulation and accumulation of soluble TNF-R1 in supernatants were in excellent correlation. Following 45 minutes of IL-1 stimulation, ~52×10^3 receptors/cell were depleted from the HPMC surface. Under similar conditions, ~32×10^3 soluble TNF-R1/cell were detected in the cell supernatant. This evidence strongly suggests that shedding of TNF-R1 is the main down regulatory mechanism following IL-1 stimulation. The short elevation in TNF receptors seen in the first several minutes following stimulation could be explained by migration of internal receptor pools to the cell surface preceding shedding.

The down-regulation of both TNF cell receptors and the appearance of soluble receptors following IL-1 stimulation could negatively affect TNF activity on mesothelial cells in the peritoneum. We postulate that these negative regulatory feedbacks are targeted to restrict the inflammatory response that had been enhanced by the presence of IL-1. We have previously shown that TNF induced the synthesis of IL-1. Combined TNF and IL-1 stimulation resulted in synergistic production of IL-1 [1]. The down-regulation and shedding of receptors could serve as a balancing mechanism restricting the synergistic pro-inflammatory activity of IL-1 with TNF. The fast TNF-R1-down regulation/shedding response to IL-1 may be followed either by, or be combined with, other local or systemic anti-inflammatory mechanisms such as the release of the IL-1 receptor antagonist (IL-1-Ra), the prostaglandin E_2 (PGE_2), or various corticosteroids. These mechanisms could well function in attuning the inflammatory response.

Zemel et al have described the presence of TNFα, TNF-R1 and TNF-R2 in the peritoneal dialysate effluent of patients undergoing CAPD and have presented evidence of local peritoneal production of TNFα and sTNF-R2 during peritonitis [22]. They demonstrated that the first phase of TNF release was followed by an accumulation of sTNF-R2 from peritoneal sources. This concurs with our findings that TNF and IL-1 induce the production of sTNF-R2 by mesothelial cells. However, this does not exclude the possible release of sTNF-Rs by recruited leukocytes or other peritoneal cells.

The PKC activator, PMA, in our experimental system, as well as in other systems, was shown to be one of the most potent shedding

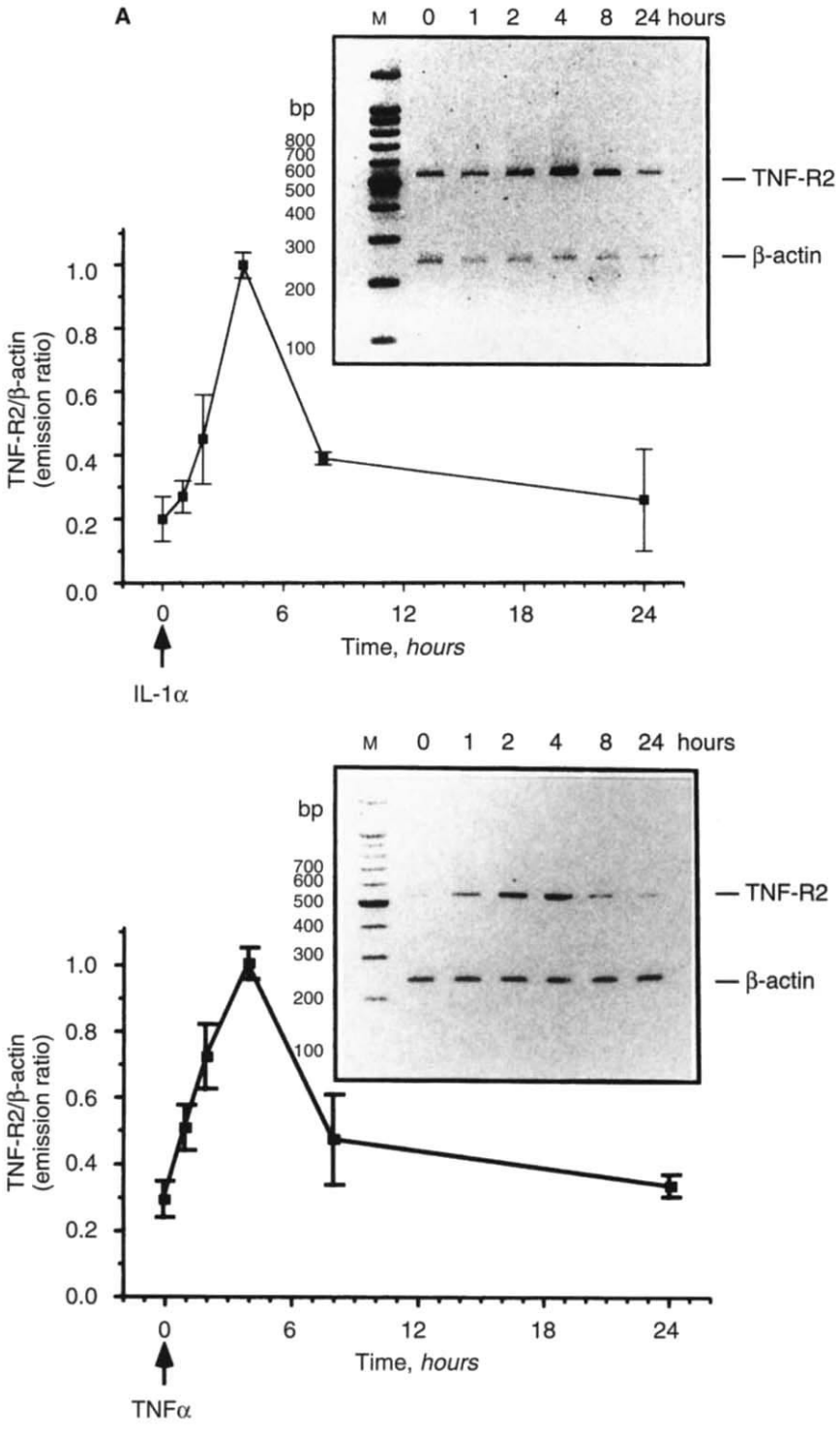

Fig. 9. *TNF-R2 RNA levels following IL-1 or TNF stimulation, time course.* HPMC were stimulated with (**A**) rhIL-1α (100 U/ml) or (**B**) rhTNFα (10 ng/ml) for various lengths of time. RT-PCR of RNA samples of activated HPMC were performed (35 cycles). On each lane, the TNF-R2 product with the respective β-actin product as the control were loaded, and analyzed on 2% agarose gel containing ethidium bromide (0.5 μg/ml). Stimulation time and the respective bands are indicated in the Figures Four additional preparations of each, IL-1 and TNF-stimulated cells from different donors were studied and yielded similar results. The ethidium bromide emission intensity of the bands was evaluated using a UVP GDS 5000 video system and software. The maximal emission ratio of each experiment was normalized to 1 and all the other samples were adjusted accordingly, allowing comparison between the experiments. The mean ± SD emission TNF-R1/β-actin ratio data of five experiments performed in duplicate is depicted in the plots.

inducers of TNF-R1. To further elucidate the role of PKC and of other PKs in signal transduction induced by IL-1α, experiments were performed using specific PK inhibitors. The PKA inhibitor, H-8, and the PKC/PKA inhibitor, H-7, significantly inhibited, in a similar pattern, both IL-1 and PMA-induced shedding. In contrast, GF, a specific PKC inhibitor, effectively blocked PMA shedding activity, but had no significant effect on shedding induced by IL-1. We thus conclude that there is a difference between the IL-1 shedding induced pathway as opposed to that of synthetic PMA. Thus, it seems reasonable to assume that PKA is involved in IL-1 shedding activity.

In conclusion, this study indicates that the main TNF receptor present on HPMC is the type-1 receptor (p55). This receptor was shedded following stimulation with IL-1α. Stimulation of the cells

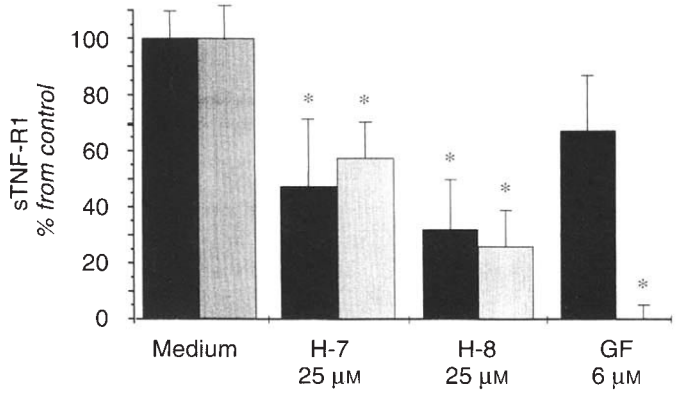

Fig. 10. *Inhibition of TNF-R1 shedding by protein kinase inhibitors.* HPMC were incubated for one hour with H-7, H-8, or GF then rhIL-1α (■, 100 U/ml) or PMA (▨, 10^{-7} M) were added for additional six hours. After incubation, supernatants were assayed for sTNF-R1 by ELISA. In each experimental plate, cells of two wells were counted for the final definition of cell number per well. The data are the mean ± SD of 5 independent experiments performed on cells from different donors. $^*P < 0.05$ experimental vs. unstimulated control.

by IL-1α or TNFα resulted in short transient enhancement of TNF-R2 RNA levels followed by the accumulation of sTNF-R2 in the supernatant. We conclude that mesothelial cells may play an important role in both the response and bio-availability of TNF during peritonitis.

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